Transcriptomic analysis of the impacts of ethinylestradiol (EE2) and its consequences for proliferative kidney disease outcome in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Freshwater fish are threatened by the cumulative impact of multiple stressors. The purpose of this study was to unravel the molecular and organism level reactions of rainbow trout, *Oncorhynchus mykiss*, to the combined impact of two such stressors that occur in the natural habitat of salmonids. Fish were infected with either the myxozoan parasite, *Tetracapsuloides bryosalmonae*, which causes proliferative kidney disease (PKD), or exposed to ethinylestradiol (EE2) an estrogenic endocrine disrupting compound, or to a combination of both (PKD x EE2). PKD is a slow progressive chronic disease here we focused on a later time point (130-day post-infection (d.p.i)) when parasite intensity in the fish kidney has already started to decrease. At 130 d.p.i, RNA-seq technology was applied to the posterior kidney, the main target organ for parasite development. This resulted with 280 (PKD), 14 (EE2) and 444 (PKD x EE2) differentially expressed genes (DEGs) observed in the experimental groups. In fish exposed to the combination of stressors (PKD x EE2), a number of pathways were regulated that were neither observed in the single stressor groups. Parasite infection, alone and in combination with EE2, only resulted in a low intensity immune response that negatively correlated with an upregulation of genes involved in a variety of metabolic and inflammation resolution processes. This could indicate a trade-off whereby the host increases investment in recovery/resolution processes over immune responses at a later stage of disease. When PKD infection took place under simultaneous exposure to EE2 (PKD x EE2), parasite intensity decreased and pathological alterations in the posterior kidney were reduced in comparison to the PKD only condition. These findings suggest that EE2 modulated these response profiles in PKD infected fish, attenuating the disease impact on the fish.

Key words: RNA-seq; Transcriptome; Rainbow trout; *Tetracapsuloides bryosalmonae*; Proliferative kidney disease; Estrogen; Anthropogenic pollution; Multiple stressor; Immune response; Metabolic processes

1. Introduction

A known biological stressor of freshwater salmonids significantly affecting both the aquaculture industry and wild fish populations globally is the extracellular myxozoan parasite *Tetracapsuloides bryosalmonae,* the etiological agent of proliferative kidney disease (PKD). In rainbow trout, *Oncorhynchus mykiss*, PKD causes an immunopathological condition mediated by an increasing number of lymphocytes and a decreasing number of myeloid cells, leading to a dysregulated B cell response and intricate interplay between t-helper th1 and th2-like cytokines (Abos et al., 2018; Bailey et al., 2017a; Bailey et al., 2017b; Gorgoglione et al., 2013). Fish can become infected at lower temperatures (<10 °C) but increasing water temperature has been demonstrated to increase disease prevalence, incidence and, severity and elevate mortalities (Bailey et al., 2017a; Burkhardt-Holm et al., 2005; Bettge. et al., 2009a; Bettge. et al., 2009b). Thus, under the growing influence of environmental change there is evidence that PKD is linked to the long-term decline of trout populations in central European countries, such as the UK and Switzerland, but also from more Nordic countries (Dash and Vasemägi, 2014; Gorgoglione et al., 2016; Kristmundsson et al., 2010; Mo and Jørgensen, 2017; Okamura et al., 2011; Wahli et al., 2002).

Given the growing impact of human activities upon aquatic ecosystems, freshwater fish suffer from chemical stressors as well as biological ones (Carpenter et al., 2011; Johnson and Sumpter, 2014). A number of pollutants are potential immunomodulators in fish. Well-known environmental contaminants with immunomodulating activity in fish include polycyclic aromatic hydrocarbons (PAHs) (Billiard et al., 2002) or polybrominated diphenyl ethers (PBDEs) (Arkoosh et al., 2015). There is now also growing evidence that estrogenic endocrine disrupting compounds (EEDCs) can have immunomodulatory effects in fish (Casanova-Nakayama et al., 2011; Milla et al., 2011). EEDCs have been reported to modulate the innate (Cabas et al., 2012; Milla et al., 2011; Seemann et al., 2016; Seeman et al., 2013) and adaptive immune response (Casanova-Nakayama et al., 2011; Cuesta et al., 2007; Rodenas et al., 2015; Saha et al., 2004; Seemann et al., 2015; Thilagam et al., 2009) in various fish species. For example, concerning the innate immune response in sea bass, *Dicentrarchus labrax*, an EEDC, 17α-Ethinylestradiol (EE2)

was shown to downregulated pro-inflammatory cytokines il1β and tnfα (Seeman et al., 2013). Regarding the adaptive immune response, EE2 exposure was shown to decrease plasma IgM levels in gilthead seabream, *Sparus aurata* (Cuesta et al., 2007). Thus, the published studies laboratory invetsigations provide evidence for EEDCs acting as immunomodulators in fish.

In view of the abovementioned information on the influence EEDCs may have on the immune system, exposure to these contaminants may modulate the response of fish exposed to the PKD causing parasite. In fact, both *T. bryosalmonae* and EE2 are considered potential factors contributing to the decline of Swiss brown trout populations over the last decades. The two stressors co-exist in Swiss rivers (Burki et al., 2006; Wahli et al., 2008, Vermeirssen et al., 2005)), what leads to the question whether their combination may lead to cumulative effect on fish populations. Nevertheless, to date only one study has examined the possible combination effects of EEDCs and *T. bryosalmonae* infection on the health status of salmonids. In an explorative study, Burki et al. (2013) exposed rainbow trout to EE2 and *T. bryosalmonae* infection and observed a dominating influence of the infection over estrogen on transcript expression of rainbow trout hepatic genes as well as parasite loads in the fish host (Burki et al., 2013). However, this study investigated an extreme disease scenario as the fish were infected at a water temperature of 18 °C, resulting in too strong pathogenesis and disease-induced mortality. This approach may have obscured possible EE2 modulation of the fish response to the parasite. EE2 was administered to fish via feeding which does not allow the exposure to relate to environmental EE2 concentrations as found in the water body. While this report provided information on the transcriptome response in the liver, it did not investigate the posterior kidney - the target organ for *T. bryosalmonae* infection.

The present study unravelled the transcriptomic response in the posterior kidney using rainbow trout exposed to either the parasite, environmentally relevant EE2 concentrations or a combination of both. Here we focused on the question whether the presence of EE2 administered in environmentally relevant concentrations via the water influences infected fish under conditions where the parasite infection still results in clinical PKD, but no elevated mortality. To this end, we performed the experiment at a temperature <15 °C, as it occurs during summer in many river

systems in Switzerland. In using this approach, we could specifically pinpoint the response the host uses to manage the parasitic infection and how it may be modulated by EE2.

We studied the EE2 modulation on PKD at a disease stage, when the parasite load of the fish kidney has gone through its maximum and started to decline (130 days post parasite infection (d.p.i)), and the fish starts to recover from infection. During the PKD recovery/resolution process the presence of the parasite will disappear from the tissue and homeostatic recovery processes will start (Bailey et al., 2019; Schmidt-Posthaus et al., 2012). In mammals estrogen can promote anti-inflammatory responses and wound healing, hence these processes may be enhanced in infected fish exposed to EE2 (Ashcroft and Ashworth, 2003; Ashcroft et al., 1997; Ashcroft et al., 2003). Therefore we hypothesised that EE2 exposure will enhance rather than supress the host response to parasite infection. More specifically, the aims of this study were to 1) obtain a broader overview on the host response to *T. bryosalmonae* infection (using RNA-seq in contrast to pre-selected genes via RT-qPCR), and 2) to greater understand how the combined impact of an estrogenic contaminant (EE2) modified the host response to *T. bryosalmonae* infection.

2. Materials and Methods

2.1. Experimental design and fish exposures

The experimental design, exposures and fish sampling are as previously described (Wernicke von Siebenthal et al., 2018). Rainbow trout were obtained from the cantonal fish farm of Rodi, Switzerland (Piscicoltura Cantonale Rodi, 6772 Rodi, Switzerland). Upon arrival, fish were examined for the presence of pathogens, including *T. bryosalmonae*, and they were found to be free of infectious agents. Rainbow trout were transferred to flow-through 35 l glass tanks, supplied with tap-water (at approx. 1 l/m), with constant and 12 h light/12 h dark photoperiod. Water temperature was kept at 12.9 °C (±1.22°C).

After a 28-day acclimation fish were separated into four experimental groups (run in duplicate tanks, 122 fish per tank at the start of the experiment): two experimental groups were exposed to environmentally relevant concentrations of water-borne 17α-ethinylestradiol (EE2 purity ≥98%) (Sigma-Aldrich, Buchs, Switzerland) exposure

(5.5 ng/l EE2) and two were not. Estradiol in Swiss midland rivers were previously assessed to range from 0.3 to 7.0 ng/l (Vermeirssen et al., 2005), thus the applied concentration can be considered environmentally relevant.

EE2 was dissolved in molecular biology purpose ethanol (purity ≥ 99.8%) (Merck, Darmstadt, Germany), and then diluted in the appropriate volume of water in a 20-l glass beaker with a magnetic rotor on the bottom to mix the solution. The solution was then pumped into the fish tanks through inert tubes (Flow Tubing, Pharamed, 4.0 mm ID, Gilson AG, Mettmenstetten, Switzerland) using peristaltic pumps (Minipuls3®, Gilson AG, Mettmenstetten, Switzerland). The flow rate was adjusted to achieve the targeted EE2 concentration in the fish tanks. The actual water inflow was controlled using Flow Rotameters (Rota Yokogawa, Wehr, Germany). The concentration of EE2 in the tanks was confirmed via water samples throughout the duration of the experiment using a competitive EE2 Elisa Kit (Ecologiena, Tokyo, Japan) (Wernicke von Siebenthal et al., 2018).

After two weeks of EE2 treatment, carried out to ensure the fish were in an estrogenic condition before parasitic exposure, one of the experimental groups exposed to EE2 and one not were then exposed to the parasite *T. bryosalmonae*. Parasite exposures were performed as previously reported in our lab (Bailey et al., 2017a). This parasite exposure still ensues in clinical PKD, but through eliminating mass mortality and continuous infection, we can precisely evaluate the physiological response the host uses to manage infection.

Consequently, the present study consists of the following four experimental groups 1) CTRL (no EE2, no parasite); (2) PKD (exposed to parasite only); (3) EE2 (exposed to 5.5 ng/L of EE2 only); (4) PKD x EE2 (exposed to 5.5 ng/L EE2 and the parasite). All treatments were performed in duplicate. All procedures were carried out according to the Swiss legislation for animal experimentation guidelines under license number BE102/16.

2.2. Fish sampling

For the present study, we used fish sampled at 130 d.p.i. Fish were euthanized using MS222 (150 mg/l buffered 3-aminobenzoic acid ethyl ester, Argent Chemical

Laboratories). Three fish (biological replicates) were sampled from each experimental group and used for all procedures. The length and weight of each fish was recorded and after blood collection, the posterior kidney was removed and weighed. The posterior kidney was then cut lengthwise. One half of the tissue was placed in a tube containing 1.5 ml TRI Reagent (Sigma-Aldrich, Switzerland) lysed and stored at -80 °C for future DNA / RNA extraction. The other half of the kidney was fixed in HistoChoice (Sigma-Aldrich, Switzerland) for 3 h at RT (room temperature) and then subsequently transferred to an ascending series of EtOH concentrations prior to paraffin embedding for histological procedures.

2.3. Pathological examination

After routine processing and paraffin embedding, kidney sections of 3–5 µm thickness was prepared on SuperFrost Plus positively charged glass slides (Thermo Fisher Scientific, Basel, Switzerland). The slides were then stained with haematoxylin and eosin (H&E) for histological assessment (Luna 1968). Histopathological alterations throughout the posterior kidney were examined, these included infection degree (presence and distribution of parasites), tissue proliferation and presence of fibrous tissue using an index previously described Bailey et al., (2018) modified from Schmidt-Posthaus et al., (2012) when assesing PKD histopathology. All parameters were scored 0–6. The degree of tissue was scored as: 0 (none), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), or 6 (severe) as. This scoring system was used to compare all experimental groups. Pertaining to infection degree: 0 indicated no parasites, whereby a 6 specified an abundance of parasites throughout the field of view. Relating to the presence of fibrous tissue: fibrous tissue is an indicator of the tissue regeneration process that is stimulated to recover/regenerate organ structure and function during the resolution of infection. Its presence has been reported in rainbow trout recovering from PKD (Schmidt-Posthaus et al., 2012). Owing to our late stage of infection analysis, we expected fibrous tissue to be observed in infected samples; hence, presence of fibrous tissue was also scored from 0–6 using the same index as described above for tissue proliferation (Schmidt-Posthaus et al., 2012). In addition, a more general toxicology pathological assessment was performed for all groups to assess any adverse impacts of the EE2 exposure. One slide was assessed per fish. To further understand the impact of the different experimental treatments' posterior

kidney somatic index (posterior kidney weight/by total body weight x 100), Fulton's condition factor ($K = W\times L^{-3} X\ 100$) and body weight were compared between all groups.

2.4. DNA isolation and qPCR for determination of parasite intensity

To determine the parasite intensity of infected fish genomic DNA was isolated from the homogenized rainbow trout posterior kidney fraction as previously described (Harun et al., 2011). DNA was eluted in 30 µl of EB buffer (Qiagen, Basel, Switzerland) and stored at −20 °C until qPCR was carried out. qPCR was performed targeting *T. bryosalmonae* 18 rDNA (Acc. N.: AF190669) as previously described (Bettge et al., 2009a), using an Applied Biosystems 7500 analyser (Applied Biosystems, Rotkreuz, Switzerland). qPCR was performed using PKD primers and the TaqMan probe as earlier described in our lab for the rainbow trout–*T. bryosalmonae* model using an Applied Biosystems 7500 analyser (Applied Biosystems, Rotkreuz, Switzerland) (Bettge. et al., 2009a). The qPCR was carried out in a final volume of 20 µl containing 1X TaqMan universal Master Mix (Applied Biosystems, Switzerland), 0.5 µM of each primer (PKDtaqf1: 5′-GCGAGATTTGTTGCATTTAAAAAG-3′ and PKDtaqr1: 5′-GCACATGCAGTGTCCAATCG-3′), 0.2 µM of the probe PKD (5'-CAAAATTGTGGAACCGTCCGACTACGA-3′) (18S rDNA gene of *Tetracapsuloides bryosalmonae*: Genbank Accession No. **AF190669**) labelled with FAM-TAMRA, 1X of IC DNA (TaqMan Univ. MMix w Exog IntPostC, Applied Biosystems), and 2 µl of template DNA. Standard curves were formed for each qPCR cycle using plasmids encompassing the amplified fragment. For every plate, five logs of plasmid dilution standards were amplified (from 10^6 - 10^2 copies). Parasite intensity in the posterior kidney (DNA copy number/g of kidney) was determined as the average parasite quantification (over the duplicate qPCR reactions) in the DNA elution, divided by the extracted kidney weight in grams (g).

2.5. RNA isolation and sequencing

RNA was isolated from the posterior kidney of three independent biological replicates from each experimental group taken at 130 d.p.i. RNA isolation and sequencing were carried out by ZF genomics. RNA was isolated using the Trizol method. The quality of all biological replicates was tested by the Agilent RNA 6000

Nano Kit, using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany) according to the manufacture's guidelines. To proceed to sequencing a sample had to exceed the quality control (QC) requirements: RNA yield >2 ug, A260/280≥1.8, and RNA Integrity Number (RIN) ≥7.0. RNA libraries were constructed and sequenced on the Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). Raw reads were stored as FastQ files. FastQ files analysis and all ensuing bioinformatics were performed by the Interfaculty Bioinformatics unit at the University of Bern, CH.

2.6. Bioinformatics, gene ontology of differential gene expression analysis

The quality of the RNA-seq data was assessed using fastqc v. 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and RSeQC v. 2.6.4 8 (Wang et al., 2012). Sequencing reads were aligned to the available rainbow trout genome (Berthelot et al., 2014) using HiSat2 (Kim et al., 2015) and quantification was performed using featureCounts (Liao et al., 2013). Differential expressed gene (DEGs) analysis between samples exposed to the different stressors (PKD, EE2 and PKD x EE2) and the control samples was completed using the bioconducter package DESeq2 (Love et al., 2014). The Benjamini Hochberg false discovery rate (FDR) correction was applied with the criteria for identifying DEGs an adjusted p value <0.05 (Benjamini and Hochberg, 1995). No threshold of log-2-fold changes was applied due to low amount of DEGs in EE2 only group.

Gene ontology analysis for the list of DEGs was performed to identify their prevalence in biological processes (BPs) with Enrichr (http://amp.pharm.mssm.edu/Enrichr/), with the Fisher exact test P-value set to <0.01. Enrichr, as per available all pathway analysis tools, is a database based on human genes and pathways and does not contain rainbow trout information. This analysis was therefore performed after converting the rainbow trout genes into their human equivalents. BPs were visually summarized using REVIGO (http://revigo.irb.hr/). For the interpretation of biologically relevant genes, we individually inspected the DEGs and placed genes into three key broad functional categories of interest using the PANTHER (protein annotation through evolutionary relationship – using the model organism zebrafish *Danio rerio*) classification

system (http://www.pantherdb.org/) and current literature. The categories were 1) Immune response related 2) Inflammation associated i.e. genes involved in apoptosis, autophagy and extra cellular matrix (ECM) components and 3) Metabolic processes. Genes were found with functions outside of these categories as well as the entire full datasets of DEGs are available in the supplementary material (Supplementary Table S1 A-C).

2.7. Statistical analysis

Here we report on and test statistically the samples used for sequencing, these were the same samples that were used for the determination of pathological assessment, posterior kidney somatic index, body weight, condition index and parasite intensity this resulted in a sample size of n=3 per condition. The rationale for this was to enable a comparison between the results of these proxies with the transcriptomic data allowing us to provide both a molecular and organism-based assessment of the host responses to the stressors. Hence, if we included extra samples in the proxy assessment, we would not have the sequencing data to make the comparison. The differences between experimental groups were tested for using a one-way ANOVA and significant differences revealed with the post-hoc test of Dunnett's. Data failing normality tests and displaying heterogeneity of variance was tested statistically applying the non-parametric Kruskal Wallis ANOVA on ranks, and Dunn's non-parametric multiple comparison tests to reveal differences. The differences between infection groups in parasite intensity (PKD vs PKD x EE2) were tested for significant differences using a t-test. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, CA, USA) or in SigmaPlot 12.0 (Systat Software, San Jose, CA, USA) and graphically presented with GraphPad Prism 5. Significance was set at $P< 0.05$. Heatmaps were created using R (Team, 2013).

3. Results

3.1. Organism-level assessment

None of the treatments significantly increased mortality in comparison to controls (experiment average - 6 ± 2.7% (Mean± S.E)). The EE2 exposure did not induce any pathological changes, whereas the parasite induced alterations consistent with PKD infection. Semi-quantitative scoring of the histological responses confirmed that the PKD only treatment had a higher score in both presence and distribution of

parasites and tissue proliferation than the PKD x EE2 treatment (Fig 1a). One of the histopathological features assessed in the trout kidney was the presence of fibrous tissue, which is generally associated with the recovery phase towards PKD resolution. No fish in the PKD-only group was found with increased fibrous tissue (score 0). In the PKD x EE2 group, only one individual displayed increased presence of fibrous tissue (score 2). In the uninfected control groups, *T. bryosalmonae* was undetectable (neither by histology or qPCR), thus neither tissue proliferation nor fibrotic changes were observed (Fig. 1a-b). No significant differences were found between the experimental groups regarding the posterior kidney somatic index and Fulton's condition factor. The weight of fish in parasite exposed groups was markedly lower than those not exposed to *T. bryosalmonae* with a significant decrease in body weight in the PKD-only group in comparison to the respective CTRL group (Fig. 2a-c). Parasite intensity was lower in the PKD x EE2 treatment, but no significant differences were found between the infection groups (Fig. 1b).

3.2. Numbers of differentially expressed genes

A number of uncharacterised DEGs in line with the study criteria but with no published symbol or determined orthologue, i.e. genes that are constructed as LOC were identified. However, as no functional information exists on these genes, they are not further discussed. This resulted with 280 (PKD), 14 (EE2) or 444 (PKD x EE2) DEGs significantly up or downregulated. Of these, the infection groups (PKD and PKD x EE2) shared 187 DEGs (Fig 3). However, the PKD and EE2 groups shared no DEGs. The number of DEGs was much greater in the multiple stressor group, although the ratio of upregulated DEGs to downregulated DEGs was comparable in the PKD and PKD x EE2 groups.

3.3. Gene ontology enrichment analysis of biological processes

The ten most significantly upregulated and downregulated gene ontology (GO) classes are shown for each exposure group (Fig. 4). In both infection groups (PKD-only and PKD x EE2) the top two BPs observed from the significantly upregulated and downregulated DEGs relative to the unexposed controls were the same (upregulated - fatty acid oxidation and fatty acid catabolic processes, downregulated - rRNA processing and ribosome biogenesis) (Fig. 4). Concerning GO classes enriched with downregulated DEGs in the PKD group, the major pathways were

associated with RNA processing and/or synthesis and chromatin organisation (Fig. 4). In the PKD x EE2 the BPs identified from the downregulated DEGs included those involved in RNA processing and/or synthesis but also several immune regulation pathways such as positive regulation of leukocyte cytotoxic cell processes and various natural killer cell positive regulation pathways. Collectively, in the infection groups many key BPs were shared, however, there seemed to be a greater amount of immune response pathways regulated in the PKD x EE2 condition. Concerning the EE2 group, surprisingly, the most significant downregulated BP was cellular response to organic substance (any process that causes a change in state or activity of a cell in terms of movement, secretion, enzyme production, gene expression, etc. as a result of an organic substance stimulus). Moreover, in this group the top 10 BPs seemed to have a greater diversity in contrast to the other experimental groups, probably due to the low amount of DEGs identified in this group.

The DEG enrichment analysis was supplemented using REVIGO (Fig. 5). The subsequent scatter plots show the cluster representatives (i.e. terms remaining after the redundancy reduction) in a two-dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities (Supek et al., 2011). This analysis further confirms that metabolic processes in both infection groups were the most dominant BPs (Fig. 5). Concerning the EE2 condition owing to the low amount of DEGs (14) and their diversity after removing redundant GO terms the REVIGO enrichment analysis resulted in no cluster representatives.

3.4. Biological interpretation of transcriptomic changes

Here is a summary of the significant up or downregulated DEGs found in the present study system. The list of DEGs was checked for biological interpretation focusing on three broad categories of interest based on GO BPs from web-based tools (PANTHER) and current literature. The chosen categories were: 1) Immune response related 2) Inflammation associated, i.e. genes involved in apoptosis, autophagy and extra cellular matrix (ECM) components and 3) Metabolic processes. In the following sub-sections, we work through each experimental group reporting the DEGs identified using the reported experimental criteria. A full list of all the genes

identified in this study and supporting data is available in the supplementary material (Supplementary Table S1 A-C).

3.5. PKD group

3.5.1. Immune response related

Overall, 36 of the DEGs were linked directly to the immune response. Of these 36, intriguingly only one was upregulated (sigirr) while the other 35 were downregulated. Concerning the innate immune response, no markers of myeloid lineage were among the DEGs. Several chemokines, ccl4 (chemokine (c-c motif) ligand 4), cxcf1a and ccl13 (Small inducible cytokine A13) were downregulated. Regarding cytokine gene expression modulation, the signal transducer and activator of transcription 3 (stat3), which acts as a key regulator of cytokine signalling pathways, was significantly downregulated, as were several interleukins involved in a broad range of host processes. These included il-1ra (interleukin-1 receptor antagonist) and il-1rii (interleukin-1 -receptor two). il-1ra inhibits il-1, and its pro-inflammatory activities (Zou and Secombes, 2016). However, not just pro-inflammatory cytokines associated genes were downregulated compared to the control group, but also il-6rα (interleukin-6 receptor alpha. High level expression of il-6ra is critical for il-6 responsiveness, which has both pro and anti-inflammatory properties (Costa et al., 2011; Zou and Secombes, 2016). In addition, il4/13a, a th2-like signature cytokine that has a role in increasing the amount of IgM secreting B cells was downregulated (Wang et al., 2016) (Fig. 6b). This expression pattern corresponds with the downregulation of all the T and B cell associated DEGs of the PKD only group in this study (Fig. 6c). The only significantly upregulated immune response related gene was sigirr, which encodes for the single Ig il-1-related receptor and is suggested to acts as a negative regulator of immune processes in mammals (Campesato et al., 2017).

3.5.2. Inflammation associated: Autophagy, Apoptosis and ECM components

pink1 (PTEN-induced kinase 1) a molecule involved autophagy processes in fish (Zhang et al., 2017) was significantly upregulated. Whereas birc5 (baculoviral inhibitor of apoptosis repeat-containing 5) and cflar (CASP8 and FADD like apoptosis regulator), genes involved in aspects of apoptosis, were significantly downregulated (Fig. 7). Regarding molecules linked to ECM components, three

DEGs were found; only one of these, mmp16 (Matrix Metallopeptidase 16) - was significantly upregulated (Fig. 7). MMPs are a major group of proteases that are important for ECM degradation in fish (Pedersen, et al., 2015).

cd63 and cd44, which were considered as ECM markers were both downregulated. cd63 is a known tetraspanin family member in rainbow trout (Castro et al., 2015) and cd44 has been reported as a tetraspanin associated protein in mammals (Zou et al., 2018) (Fig. 7). Tetraspanins in mammals modulate the function of proteins involved in all determining factors of cell migration including cell–cell adhesion, cell–ECM adhesion, cytoskeletal protrusion/contraction, and proteolytic ECM remodelling (Jiang et al., 2015).

3.5.3. Metabolic processes

In stark contrast to the DEGs reported in the immune response section, we observed significant upregulation of all the genes associated with metabolic linked function in the PKD only group. Functions of these genes include metabolic generalists (dcxr-dicarbonyl and l-xylulose reductase), molecules with roles in the fatty acid metabolism (acsf2 - Acyl-CoA synthetase family member 2, mitochondrial, pecr-peroxisomal trans-2-enoyl-coa reductase), histidine catabolism (amdhd1-amidohydrolase domain containing 1) and gluconeogenesis (pck1-phosphoenolpyruvate carboxykinase 1). Pck1 was the most strongly upregulated gene which is a key regulator of gluconeogenesis (Fig. 8).

3.6. EE2 group

Only 14 DEGs were found in the EE2-only exposed group, when compared to the untreated control, thus results were analysed as a single entity (Fig. 9). Concerning immune genes il-21r (interleukin 21 receptor) was downregulated. ddit4 (DNA-damage-inducible transcript 4), a molecule that in mammals is involved in varied biological processes such as antiviral activities and apoptosis was upregulated. cyp1b1 (Cytochrome P450 1B1), which belongs to the Cytochrome P450 superfamily of enzymes and functions to metabolize potentially toxic compounds was downregulated (Uno et al., 2012). sult1a4 (sulfotransferase family 1a member 4) and prodh2 (proline dehydrogenase 2) genes, which have metabolic functions were significantly upregulated. Other upregulated genes included amh (muellerian-

inhibiting factor), which has a role in the fish gonadotropin-releasing hormone receptor pathway and oplah (5-oxoprolinase, atp-hydrolysing), which has a role in ATP binding. Other downregulated genes were fd6d (putative delta 6-desaturase-), and genes involved in diverse pathways, from transmembrane trafficking (pik3r4 - phosphoinositide-3-kinase regulatory subunit 4), transcriptional regulation (jun- jun proto-oncogene), cytoskeleton junctions (ptpn4-protein tyrosine phosphatase non-receptor type 4), biomineralization (fam20a- golgi associated secretory pathway pseudokinase), RNA binding (dhx30- dexh-box helicase 30), and protein ubiquitination in mammals (kbtbd3- Kelch repeat and BTB (POZ) domain-containing 3).

3.7.PKD x EE2

3.7.1. Immune response related

In the stressor combination group, 62 DEGs were associated with the immune response. Out of these 7 were upregulated and 55 downregulated. Regarding the innate immune response, markers of the myeloid cellular lineage; slc11a1 (formerly known as natural resistance-associated macrophage protein1, nrampα) and mcsf1 (macrophage colony stimulating factor precursor) were significantly upregulated. Likewise, kit (kit proto-oncogene receptor tyrosine kinase), the mast/stem cell growth factor receptor was also significantly upregulated. However, several chemokines including ccl4, cxcf1a and ccl13 were downregulated (Fig. 10a). Regarding genes associated with the complement system, cfi (complement factor 1) was upregulated and actually had the strongest expression of any of the immune genes in this experimental group, although other molecules involved in the complement masp1 (mannan binding lectin serine peptidase 1) which functions in the lectin pathway of complement and c4 (Complement component 4) were downregulated (Fig. 10b).

Regarding cytokine gene expression modulation, there were 15 DEGs identified all of which were downregulated. In this group, we also included nmi (N-Myc (And STAT) Interactor) as it closely interacts with the cytokine mediator stat1 (also grouped here). Aside from nmi, 13 interleukins involved in a wide range of innate and adaptive processes were significantly downregulated (Fig 10c). In addition, several genes involved in a diverse array of immune processes such as interferons (irf8 and irf-1) and toll-like-receptors (tlr7 and tlr22) were significantly downregulated.

Concerning the expression patterns of genes involved in adaptive immune processes, for the B cell lineage only bcl6b (B cell CLL/lymphoma 6B) was significantly upregulated. In fact, no other genes that were considered solely B cell specific markers i.e. only molecules, which could be considered to be associated with general lymphocyte processes (both B and T cells related) were differentially expressed in this group (Fig. 11).

A large number of genes (17) encoding with diverse aspects of T cells functional activity were observed in this study. Out of these genes, only notch was upregulated. Notch is part of the notch-signalling pathway; a highly conserved cell signalling system that in mammals is involved in multiple processes and has been suggested to be is essential for initial commitment to the T cell lineage and may function together with signals from the pre-tcr and the tcr to regulate subsequent steps of T cell development (Deftos and Bevan, 2000; Guidos, 2006). The genes downregulated that were markers of different T cell processes included many well-studied molecules in fish immunology: tbet, cd8a, foxp3-1, foxp3-2, cd3e and mhcII. A gene not grouped to any of the mentioned immune response pathways here: sigirr, as in the PKD only group, was also significantly upregulated (0.8 in PKD x EE2 vs 0.8 in PKD only - log 2fold change).

3.7.2. Inflammation associated: Autophagy, Apoptosis and ECM components

DEGs involved in the autophagic processes in the combined stressor group included ddit4l (DNA Damage Inducible Transcript 4 Like) and ddit4 which had different expression profiles, with ddit4l downregulated and ddit4 upregulated. While all of the transcripts associated with apoptosis activity were downregulated (cflar, birc5, casp8 - - caspase 8, sap30bp - sap30 binding protein). The transcriptional signatures of the ECM component linked DEGs in this experimental group were similar to the PKD only group in that cd63 (-0.69 in PKD x EE2 vs -0.74 in PKD only - log 2fold change) was downregulated and mmp16 was upregulated (0.78 in PKD x EE2 vs 0.7 in PKD only- log 2fold change. However, additional DEGs that encode for ECM constitutes were also identified in the PKD x EE2 group such as (pxdn- peroxidasin, fgfr2-fibroblast growth factor receptor 2, igtb1bp1- integrin beta-1-binding protein 1 and vit-vitrin) with vit, pxdn and fgfr2 significantly upregulated. Indicating a stronger

presence of ECM components in this infection group in contrast to the other conditions (Fig.11).

3.7.3. Metabolic processes

Similar to the PKD only group, almost all of the DEGs involved in metabolic functions were upregulated. The only gene downregulated was npc2 (NPC intracellular cholesterol transporter 2) which is involved in many different biological processes of the metabolism including the cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism and the sterol metabolism. DEGs with metabolic functions that were upregulated-included pck2, which is, involved in the biological process gluconeogenesis, as well acsf2 and acaa1 (acetyl-CoA acyltransferase 1) which are involved in the fatty acid metabolism and amdhd1, which is involved in the histidine catabolism (Fig. 12).

4. Discussion

In the present study, transcriptome profiling of the rainbow trout posterior kidney was undertaken at 130 d.p.i to 1) obtain a broader overview on the host response to *T. bryosalmonae* infection (using RNA-seq in contrast to pre-selected genes via RT-qPCR), and 2) to greater understand how the combined impact of an estrogenic contaminant (EE2) modified the host response to *T. bryosalmonae* infection. We hypothesised that EE2 exposure will enhance rather than supress the host response to parasite infection. In the *T. bryosalmonae* infected fish we found a less intense immunological response in contrast to the published literature (see Abos et al., 2018; Bailey et al., 2017a; Bailey et al., 2017b; Gorgoglione et al., 2013). However, these studies used either functional or RT-qPCR approaches in contrast to RNA-seq in the present study. In the PKD x EE2 treated fish we found a more intense transcriptomic response in comparison to the other conditions in terms of the amount of DEGs upregulated in key physiological responses during *T. bryosalmonae* infection, additionally both parasite intensity and pathology were reduced in comparison to the PKD treatment. While posterior kidney somatic index, body weight and condition factor were comparable in the two infection groups. Furthermore, concerning both infection groups, many of the major BPs identified from upregulated and downregulated DEGs were shared. However, there was a greater amount of immune response related and inflammation associated genes upregulated in the PKD x EE2

group, although the regulation of metabolic genes was comparable between both infection groups. While in the EE2 group, we found a very low amount of DEGs with high diversity of BPs. Overall, in our study system the estrogen appeared to have no negative suppressive action, but a partly enhancing action on *T. bryosalmonae* infected fish.

The present study is the first of our knowledge to investigate the host response at a late disease phase during *T. bryosalmonae* infection. Here we identified genes involved in a variety of metabolic functions and tissue inflammation resolution and repair processes, not just in the PKD group but in the PKD x EE2 group as well that suggest the fish downscales its investment in the immune response and triggers the recovery/resolution process. We show in both infection groups a negative correlation: an upregulation in metabolic genes and a downregulation in immune genes. This negative correlation could indicate a trade-off of priorities at the late stage of infection whereby the host increases investments in one function (energy and intermediary metabolism) leading to decreased investments in another function (immunity). Life-history theory predicts trade-offs occur between different traits such as growth and maintenance, reproduction and immunity (Zera and Harshman, 2001). In the infection groups fish had a lower weight when compared to those not infected, thus it could be speculated that this occurred due to an investment in the immune response. At the investigated stage with parasite burden decreasing there may be a change in host priorities to address this.

Previous immunological investigations of *T. bryosalmonae* infection in salmonids has led to a description of the disease as an immunopathological condition mediated by decreasing myeloid cells and increasing lymphocytes, in addition to complex back and forth interplay between th-like subsets (Abos et al., 2018; Bailey et al., 2017a; Bailey et al., 2017b; Bailey et al., 2019; Gorgoglione et al., 2013). In the present study, relating to the PKD only treatment, our investigation performed at a late stage of infection using an RNA-seq approach resulted in a different outcome concerning the immune repertoire used by rainbow trout. The outcome was different in two dimensions, in terms of intensity, and, in terms of the mechanisms of the immune response. Many pathways previously reported to play a role in the PKD pathogenesis were downregulated or unresponsive in the present study. The less

intense immune response observed in the infection groups might indicate that the host never had to use this immune response, as the disease was not severe enough or that the reduction in infection pressure at the later time point allowed the host to shift priorities away from immunity and reallocate resources to other physiological functions. For instance, the regulation of genes that are involved infection resolution and recovery or metabolic processes. In the study by Wernicke von Siebenthal and colleagues (2018), using data from the same experiment collected at an earlier time point (90 d.p.i) demonstrated strong upregulation of il-10, blimp1 and IgM sec in both infection groups. This would confirm that a more intense immune response did occur at an earlier PKD phase. This response was consistent to previous studies of PKD as per (Bailey et al., 2017a; Bailey et al., 2017b; Gorgoglione et al., 2013). This suggests that the host switched priorities away from the immune response in the present study.

Surprisingly, in the PKD-only group there was only one immune gene significantly upregulated relative to the control, the single Ig and tir domain containing (sigirr). Concerning studies of this gene in fish, sigirr was not found in the green spotted pufferfish, *Tetraodon nigroviridis*, three-spined stickleback, *and Gasterosteus aculeatus*, or Japanese puffer, *Takifugu rubripes* genomes. Instead, a double ig il-1r related molecule (digirr) was found. In the same study phylogenetic analysis revealed that digirr was homologous to sigirr, but distinct in both protein structure and subcellular localization. *In vivo* and *in vitro* functional characterization indicated that digirr acted as a negative regulator of il-1 mediated signalling (Gu et al., 2011). In mammals sigirr is (known as il-1r8) acts as a negative regulator of toll-like and il-1 receptor family signalling, with its upregulation suggested to contribute to an impaired innate immune sensing and the development of an antitumor immune response (Campesato et al., 2017). However, as the expression of pro-inflammatory cytokines in previous PKD studies has been shown to be only transient or downregulated (Bailey et al., 2017a; Gorgoglione et al., 2013; Holland et al., 2003), sigirr expression is probably not unique to this disease stage studied here. Moreover, it could be plausible that sigirr may even be a target for parasite manipulation in early stage infection as an immune evasion tactic, given its negative modulation of the innate response and its reported role in innate immune sensing.

Concerning the lack of a stereotypical PKD dominant B cell response, at 130 d.p.i; this raises the question in the present study that the time point investigated was too late and we missed this response. Corresponding to this, parasite intensity had already plateaued at 90 d.p.i, thus the lack of a B cell response correlates to the overall reduced intensity of infection from both a host and parasite perspective. Notwithstanding, if we did not see a dominant lymphoid driven response it could be speculated that there may be some indication or homeostatic rebalancing of the myeloid arm of the immune response, given its described unresponsiveness during clinical infection, but we did not see this here. It could be that this transpired at an earlier time point or that it occurred via the downregulation or unresponsiveness of the B and T mechanisms reported here. To really address such a question would require a longitudinal approach with a greater amount of sequential data as well as the inclusion of functional data. We only found upregulation of one specific B cell marker; bclb6 occurring in the PKD x EE2 condition. bclb6 and both blimp1 are key regulators of B cell terminal differentiation in mammals. Functional characterization of these genes in fugu showed that they also regulate B-cell terminal differentiation also in fish (Ohtani and Miyadai, 2011). Taking this into account and given the reported role of blimp1 in PKD pathogenesis of both rainbow trout and brown trout in the studies by Bailey et al., in 2017 and 2019, this molecule (bclb6) may be of interest to future studies of B cell differentiation in fish.

While concerning the T cell response of interest in the PKD x EE2 was the downregulation of tbet, cd8a, foxp3-1, foxp3-2, cd3e and mhcII. While PKD mediated suppression of innate immune aspects has been reported (Chilmonczyk et al., 2002). All of these genes (apart from cd3e) have been shown to be upregulated in the rainbow trout immune response to *T. bryosalmonae* (Bailey et al., 2017a; Gorgoglione et al., 2013). Thus, the downregulation of these genes might suggest that PKD x EE2 is having a stronger immunosuppressant role than in PKD alone. This would corroborate the study by Burki et al., 2013 who found many immune genes downregulated in the liver after combined *T. bryosalmonae* infection and EE2 exposure and suggested a potential immunosuppressive action of EE2 in rainbow trout. However, a greater number of upregulated genes found in our study in the PKD x EE2 treatment suggests the fish can still mount an immune response even in the face of EE2-induced immunosuppression and as also suggested by Burki et al.,

2013 this may have actually translated into the decreased parasite intensity and reduced pathological alterations.

It might be expected that fish exposed to multiple stressors will have a more intense transcriptomic response owing to increased physiological demands in managing the combination of stressors resulting in increased pathogen burden and more severe disease. However, owing to the previous study by Burki et al (2013) that EE2 exposure of *T. bryosalmonae* infected fish resulted in decreased parasite burden we predicted that EE2 exposure will enhance rather than supress the host response to parasite infection. The results obtained here would support our prediction as the increased amount and presence of specific DEGs in the PKD x EE2 group correlated with decreased parasite intensity and reduced pathology. Though it must be pointed out, as a possible limitation of this study is that we only used three fish per experimental group in our investigation. Our results raise the question: did EE2 enhance the immune response in the PKD x EE2 group? While, it cannot be ruled out that there was a negative impact on the parasite in the present study as both positive and negative impacts of environmental contaminants on the parasite have been noted in other host-parasite systems (Krasnov et al., 2015; Romano et al., 2015). We did have more immune response genes upregulated in the PKD x EE2 group at the time point investigated. Nevertheless, given the immunopathological nature of PKD it could be disputed whether such “immunoenhancement” would benefit the host. Consequently, we do not know from the present study if an immunosuppressive response occurred at an earlier time point and if this is what caused the lower pathology in the PKD x EE2 treatment, as decreasing resistance mechanisms through immunosuppression might reduce immunopathology benefiting the host.

Several lines of evidence in our study suggest that in the PKD x EE2 condition there is a greater host investment in infection resolution/recovery. In this group, there was an increased amount of DEGs linked to ECM processes relative to the PKD-only group. This might indicate an increased investment in tissue reorganisation in the kidney, through either immunopathological or parasite-induced damages. The expression of genes involved in apoptosis was indeed seen downregulated in both infection groups. It has been well documented that programme cell death plays an

important role in inflammation resolution via clearance of immune cells. For instance, failure of apoptosis to clear such cells is a major driver of chronic inflammation (Yang et al., 2015). However, the lack of a strong B or T cell response in the infection groups might indicate that the host may have already undergone a major immune cell clearance event, which would correspond with the observed downregulated of expression patterns of these pathways in our study.

Additionally, in the PKD x EE2 group concerning the immune response related genes there was also some indication that the host has further progressed along in the recovery process, for instance through expression of M2 macrophage phenotypes that have 'anti-inflammatory', or 'pro-healing' phenotypes (Grayfer et al., 2018; Martinez and Gordon, 2014; Wiegertjes et al., 2016). In this perspective, slc11a1 (formerly known as nrampα - natural resistance associated macrophage protein alpha) and mcsf-1 were both upregulated in the PKD x EE2 infection group, thus we questioned if these molecules were playing a role in tissue resolution. slc11a1 in mammals is associated with the ability of macrophages to destroy bacterial pathogens (Vidal et al., 1993), however in fish there is no functional data concerning this gene. While mcsf-1 in fish has been associated with classical pro-inflammatory actions, it has also been shown to play a role in macrophage differentiation (Grayfer et al., 2009). Although il4/13a and il10, which are usually suggested to be M2 polarizing agents, were both downregulated in this group which would suggest the contrary (Grayfer et al., 2018; Wiegertjes et al., 2016). Interestingly in the PKD x EE2 mcsf-1 (upregulated), il4/13a and il10 (downregulated) expression patterns contrasted to those previously reported in PKD gene screenings (Bailey et al., 2017a; Gorgoglione et al., 2013). In addition, in this group kit (kit proto-oncogene receptor tyrosine kinase) also known as a mast/stem cell growth factor receptor was significantly upregulated. The presence of kit (then called c-kit) on zebrafish mast cells was shown using immunohistochemistry by Dobson et al., 2008. Therefore, expression of this molecule might indicate some involvement of these cells. In fact, fish mast cells are also suggested to play a role in wound healing in fish (Sfacteria et al., 2015). Thus, when piecing together the histology data, the reduced parasite intensity and the transcriptomic results there is a general indication that there is a greater investment in resolution/recovery processes from infection in the PKD x EE2 group.

We did not find a strong impact of EE2 on the fish not exposed to the parasite, either from the transcriptomic data or toxicologically. This response may be a tissue specific issue as it has been previously reported that EE2 has major effects on gene regulation in the liver and gonads at the selected concentrations (Shved et al., 2007). In this group, there were only 14 DEGs and only one immune gene was identified il-21r. il-21r transduces the growth-promoting signal of il-21. il-21 is a th17 cytokine and suggested to be a likely regulator of T and B cell functions. The gene has been described as an important player in the rainbow trout immune response against bacteria and viruses as well correlated with increasing parasite intensity in PKD pathogenesis (Gorgoglione et al., 2013; Wang et al., 2011). However, considering the evidence of EE2 in the literature as an immunosuppressor (Cabas et al., 2012; Cuesta et al., 2007; Milla et al., 2011; Rodenas et al., 2015; Saha et al., 2004; Seemann et al., 2016; Seemann et al., 2015; Thilagam et al., 2009) the downregulation of only one immune gene hardly accounts for immunomodulation. Surprisingly, the most significant downregulated BP was cellular response to organic substance. In line with this, cyp1b1 was also downregulated; cy1b1 is part of the cytochrome p450 (CYP450) group of genes that play an important role in catalysing oxidative metabolism of toxicants. Moreover, cyp genes are used as biomarkers of exposure to several organic contaminates found in the aquatic environment (i.e. PAHs and PCBs) (Nebert and Karp, 2008; Pinto et al., 2015; Zanette et al., 2009). In addition, it has also been suggested that estrogens appear to have a modulating effect on the expression of cytochrome P450 genes (Navas and Segner, 2000). Therefore, we would have expected that this gene or other cytochrome p450 family members might be upregulated. However, this could be explained by the fact that the fish may have adapted to chronic EE2 exposure in the present study, as ectotherms have been shown to have great plasticity to maintain key physiological processes under these stressors (Wernicke von Siebenthal et al., 2018), or on the other hand that the gene or one its isoforms are stronger expressed in other tissues as such expression site exclusivity exists for this family of genes in fish (Zanette et al., 2009).

3. Conclusion

In this study, we investigated the rainbow trout transcriptome and its relevance for the organism outcome when confronted by both a biological and chemical stressor. We demonstrated that the cumulative impact of EE2 and *T. bryosalmonae* infection

as multiple stressors resulted in pleiotropic effects cascading multiple physiological systems not observed in the other experimental groups. We observed post clinical fish show a different molecular signature concerning the immune response, and EE2 appears to enhance aspects of the PKD recovery process. We provided evidence for a different molecular signature of the host response during the PKD resolution stage in rainbow trout. In doing this, we were able to build on the existing knowledge concerning the fish response to *T. bryosalmonae* infection. Our results supported earlier studies, that while EE2 could modulate the fish physiology, it might not necessarily act as a stronger immune-suppressor when in concomitance with a chronic immunosuppressant disease.

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Figures

Fig 1. (A) Pathological assessment of the posterior kidney compromising of histopathological scores for presence of parasites, tissue proliferation and fibrous tissue. Different colour bars denote experimental groups. (B) Parasite intensity in PKD and PKD x EE2 conditions. Parasite intensity was determined using copy numbers of parasite DNA per fish standardised using the individual fish kidney weights. No pathological changes or presence of parasites was found in EE2 or CTRL groups. No significant differences were found concerning either parameter $P < 0.05$. N =3.

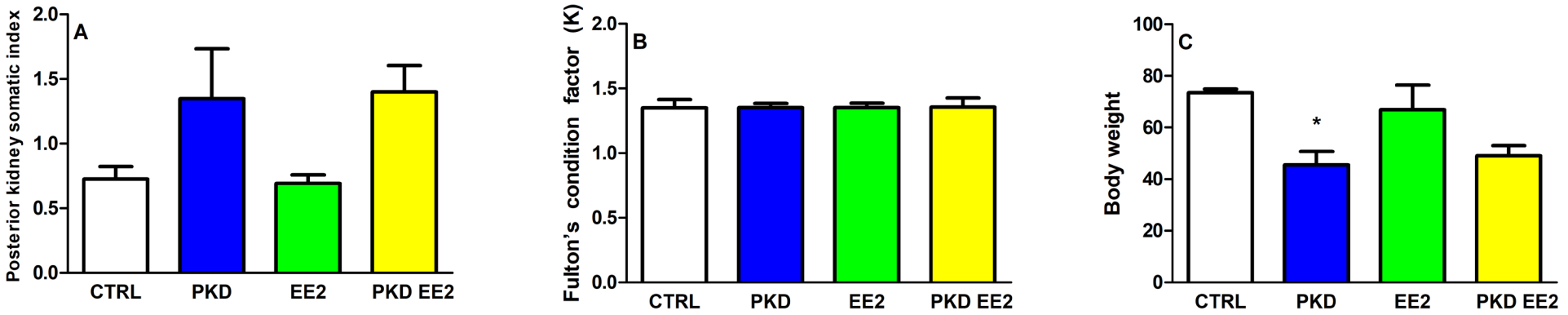

Fig 2. Bar charts showing (A) Posterior kidney somatic index scores (B) Fulton's condition factor (K) and (C) Body weight of all experimental groups. Different colour bars denote different experimental groups. CTRL = white bars, PKD = parasite exposure only (blue bars), EE2 = chemical exposure only (green bars) and PKD x EE2 = parasite and chemical exposure (yellow bars). $P < 0.05$. N =3.

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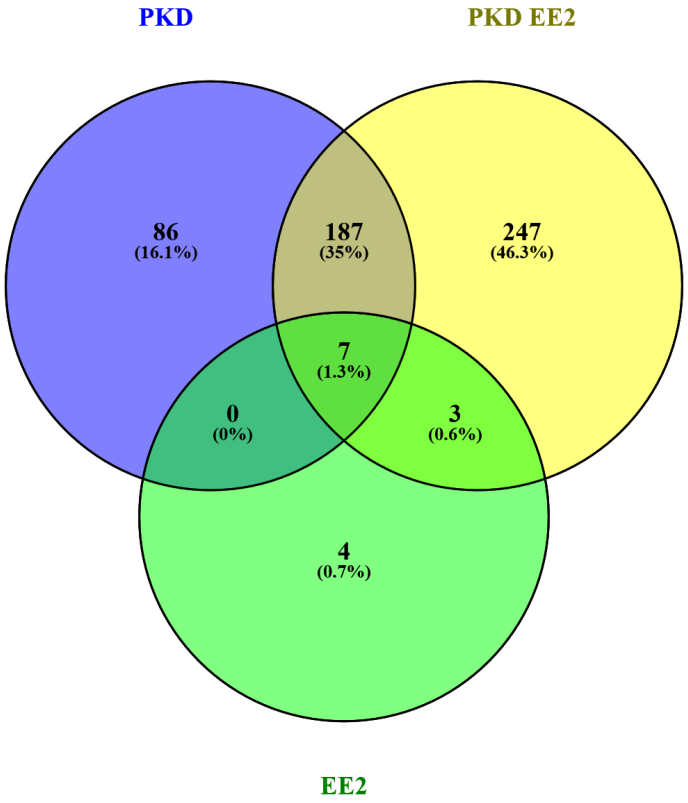


Fig 3. Venn diagram showing the number of differentially expressed genes in the different treatments and intersects between them. PKD = parasite exposure only (blue circle), EE2 = chemical exposure only (green circle) and PKD x EE2 = parasite and chemical exposure (yellow circle).

Upregulated GO classes

Downregulated GO classes

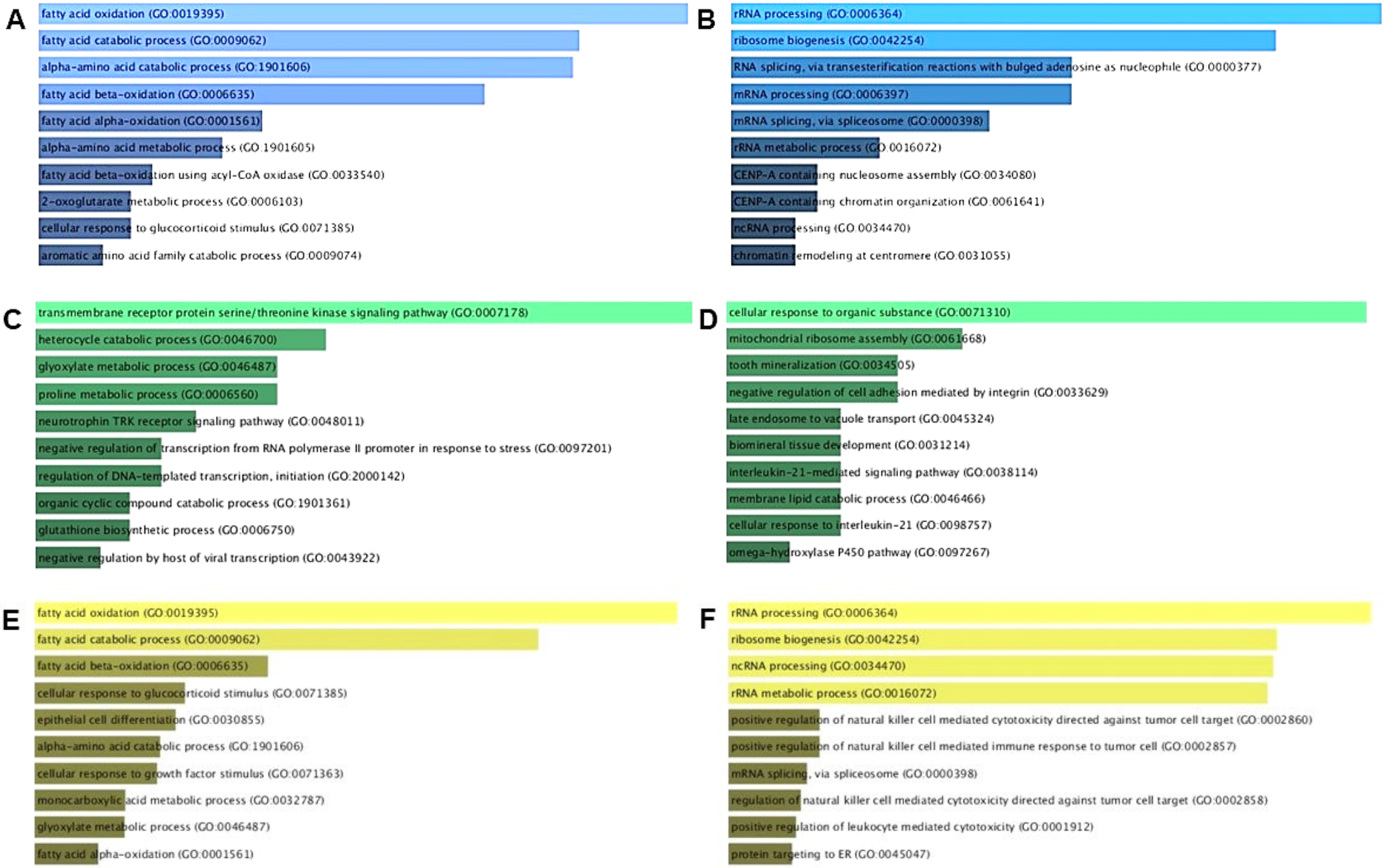


Fig 4. The ten most significantly upregulated and downregulated GO classes were classified according to their involvement in biological processes. All DEGs were subjected to an enrichment analysis via the online gene list analysis tool, *Enrichr*, are in the order of their P-value ranking. PKD = parasite exposure only (blue bars: A – upregulated, B - downregulated), EE2 = chemical exposure only (green bars: C – upregulated, D - downregulated) and PKD x EE2 = parasite and chemical exposure (yellow bars: E – upregulated, F - downregulated).

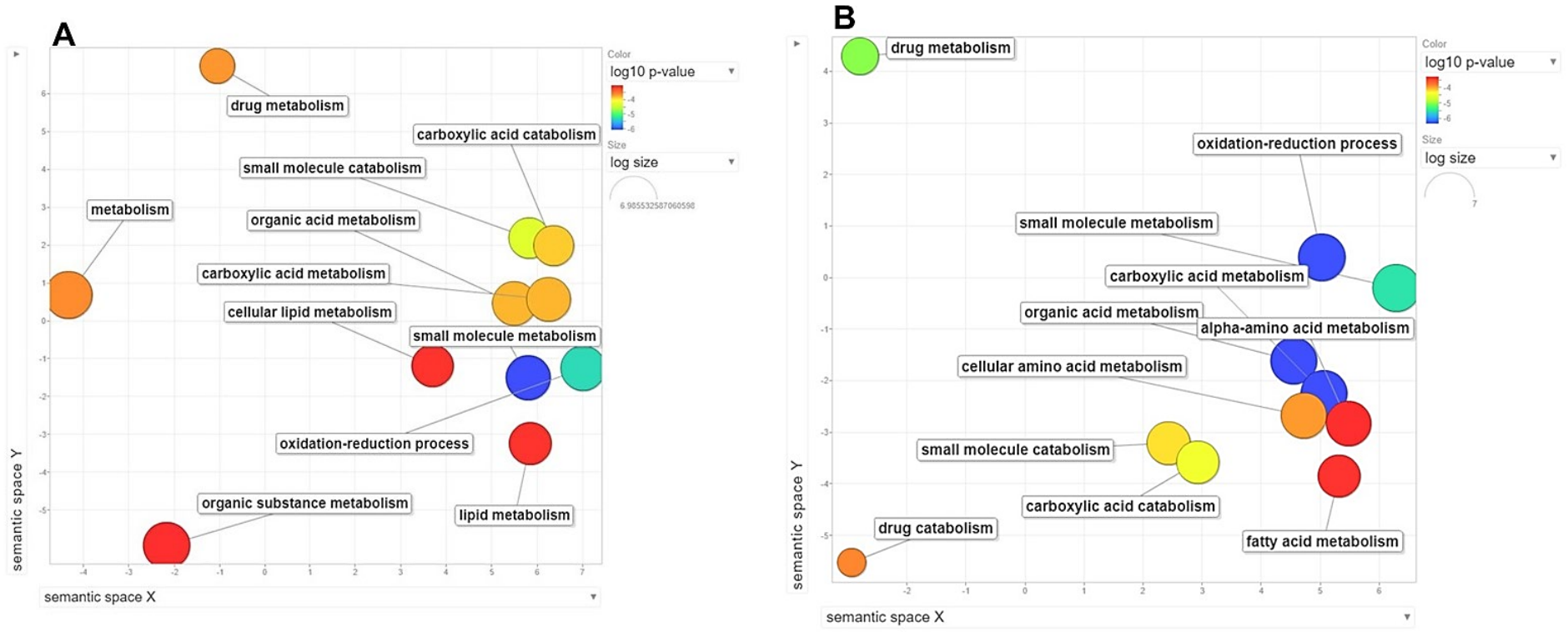
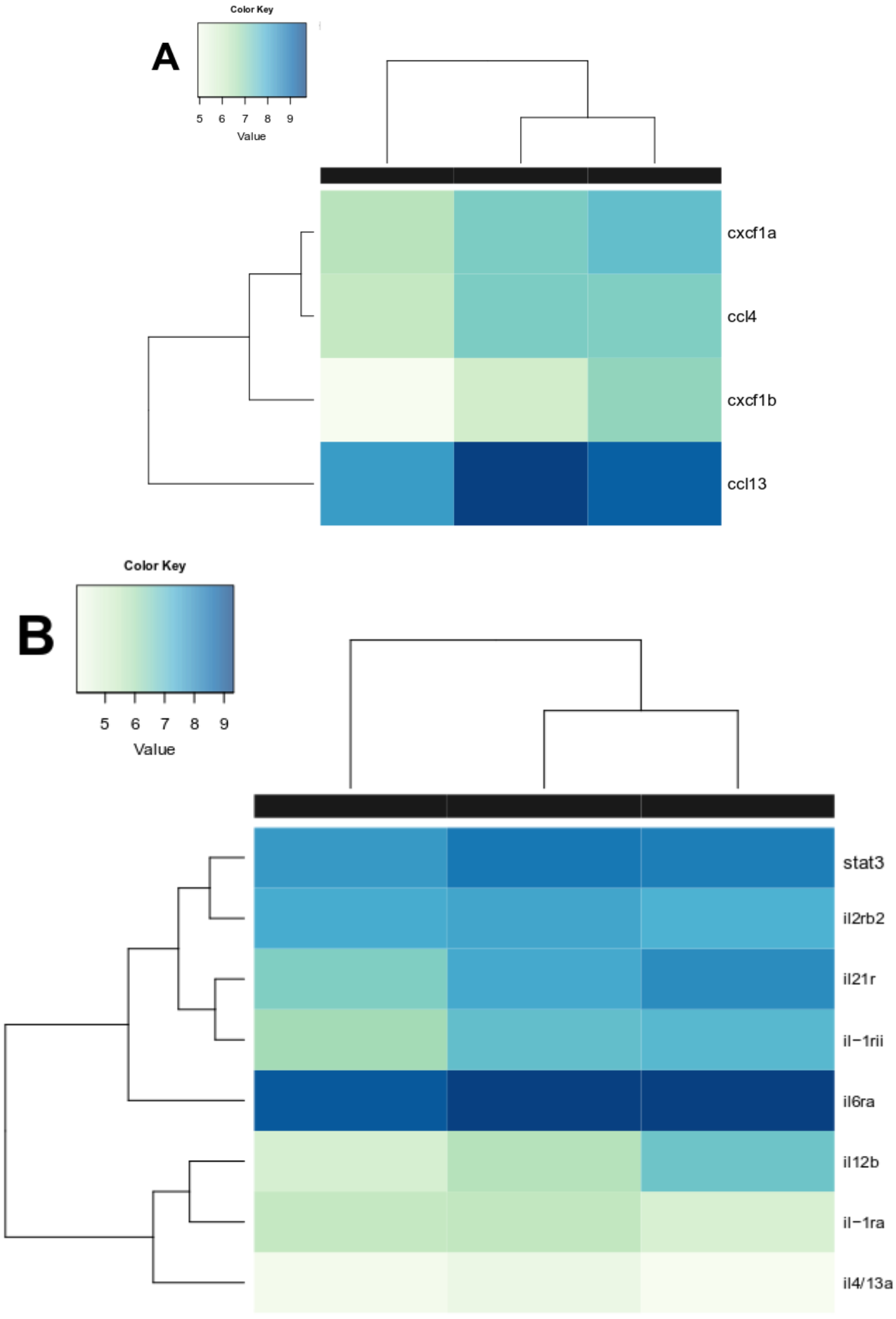


Fig 5. Results of REViGO semantic analysis of GO biological process of A) *T. bryosalmonae* infected fish and B) PKD x EE2 exposed fish. The principle is that semantically similar GO biological processes should remain close together in the plot, but the semantic space units have no intrinsic meaning. Circle colour indicates the p-value for the false discovery rates; circle size indicates the frequency of the GO term in the underlying GO database (circles of more general terms are larger; http://revigo.irb.hr/).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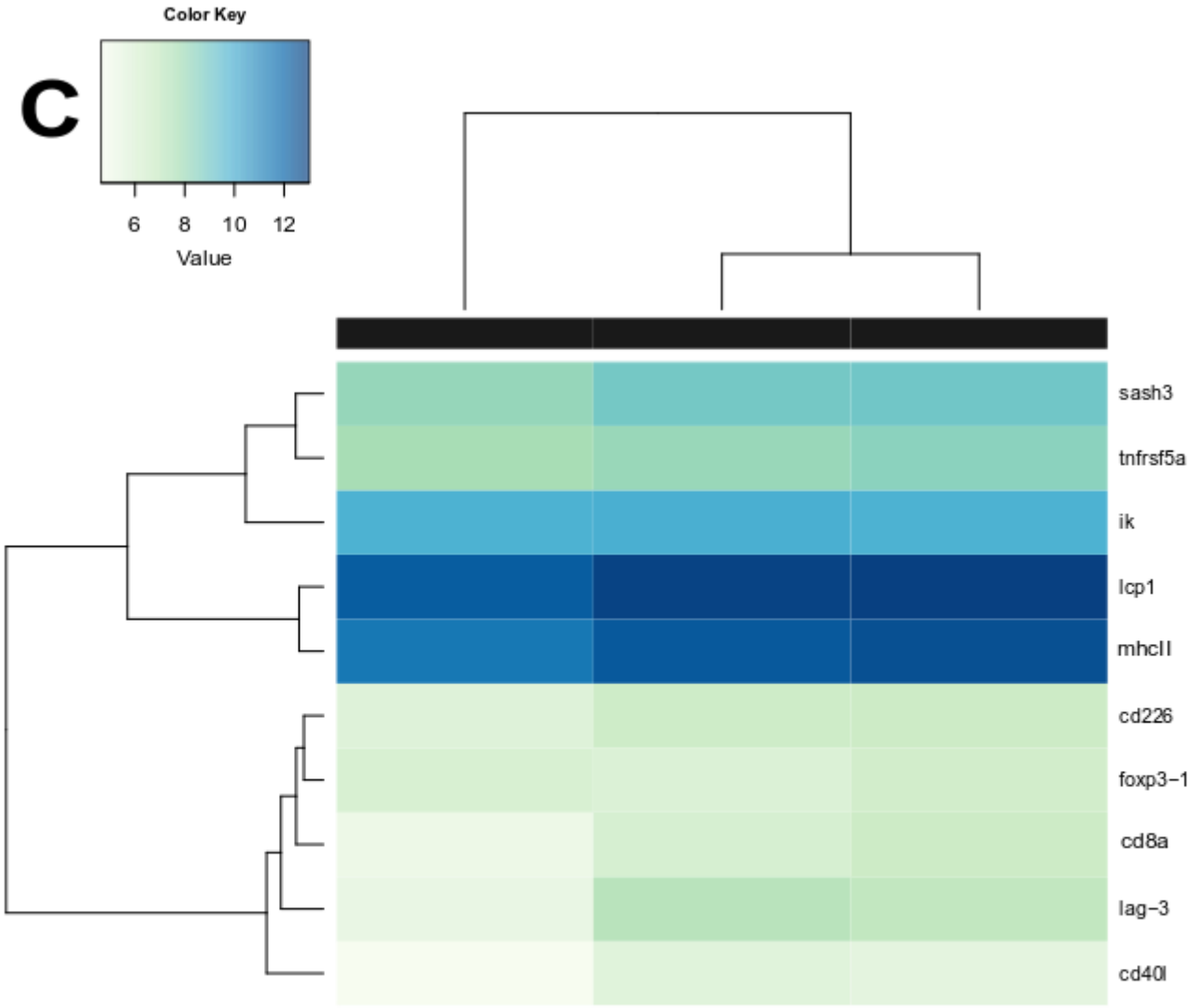


Fig 6. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of A) chemokines; B) cytokines; C) B cell and T cell related genes in rainbow trout posterior kidney of *T. bryosalmonae* infected fish. Some genes in C) exclusive i.e. involved in both B and T cell mechanisms. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

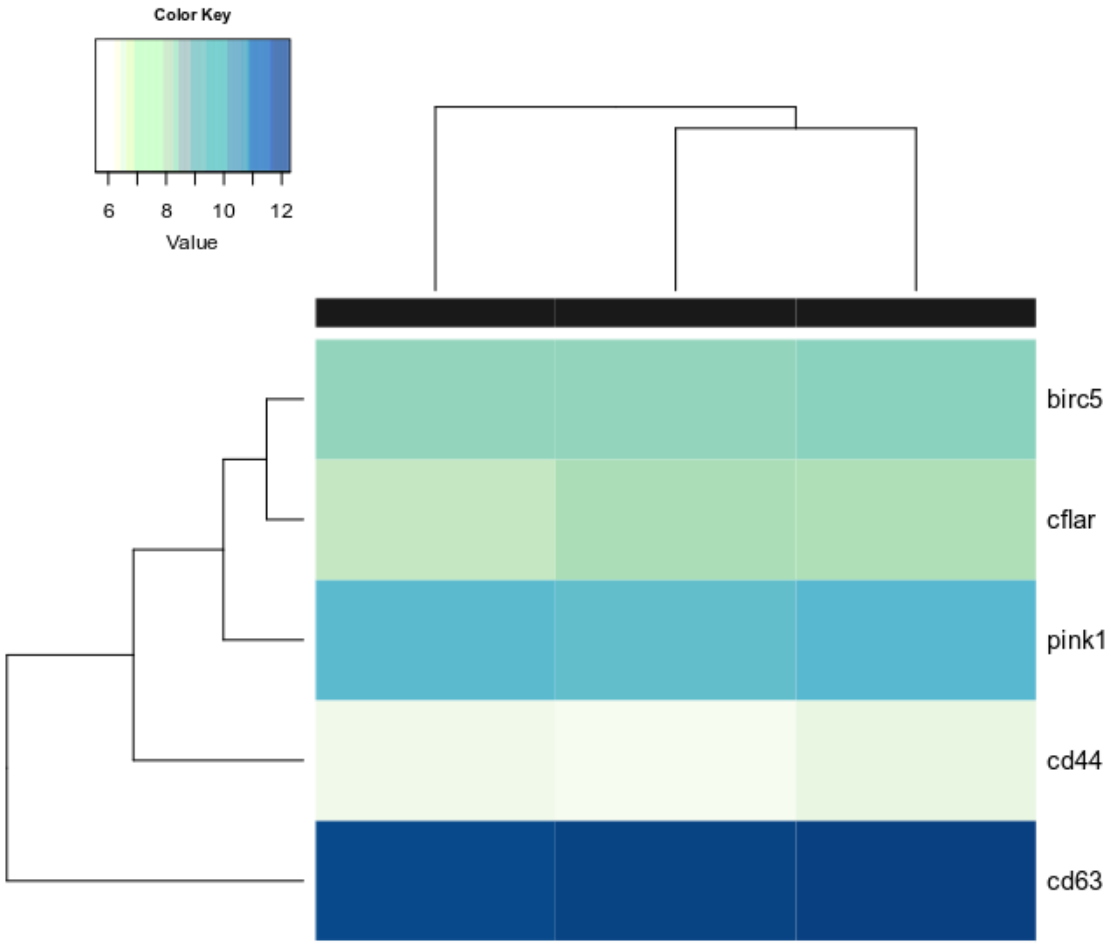


Fig 7. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of inflammation associated genes i.e. genes involved in apoptosis, autophagy and extra cellular matrix (ECM) components in the rainbow trout posterior kidney of *T. bryosalmonae* infected fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

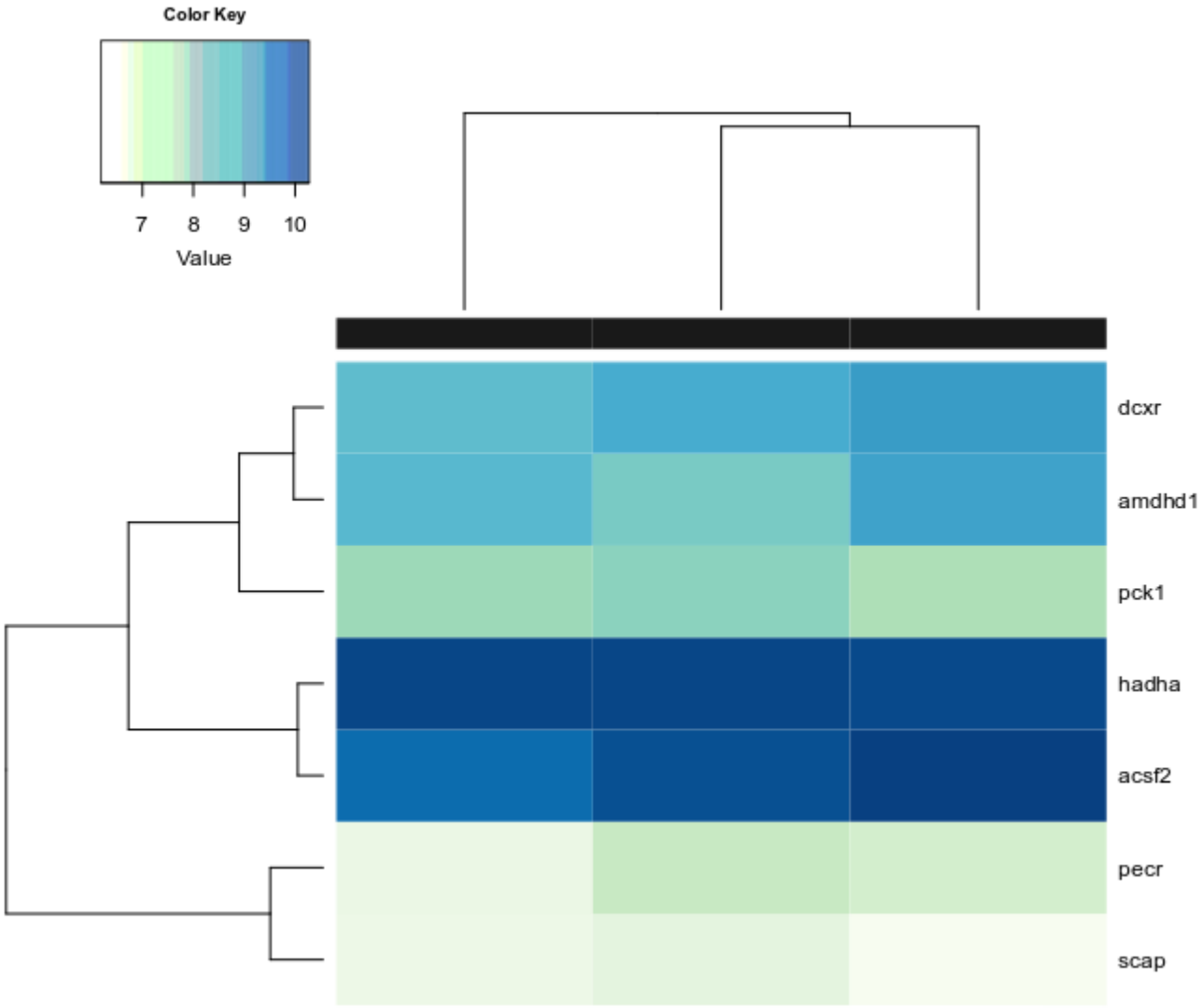


Fig 8. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of genes involved metabolic functions in the rainbow trout posterior kidney of *T. bryosalmonae* infected fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

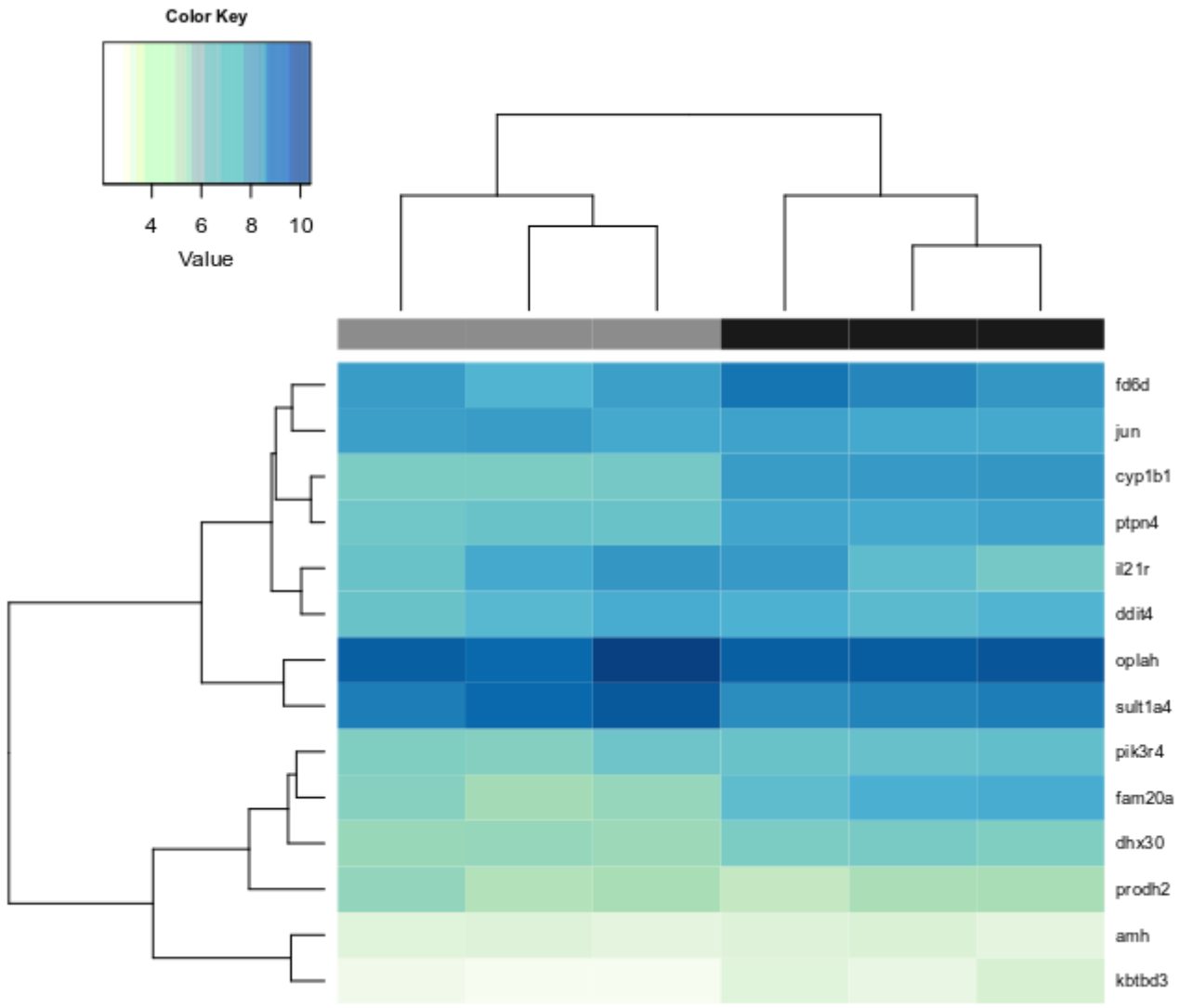
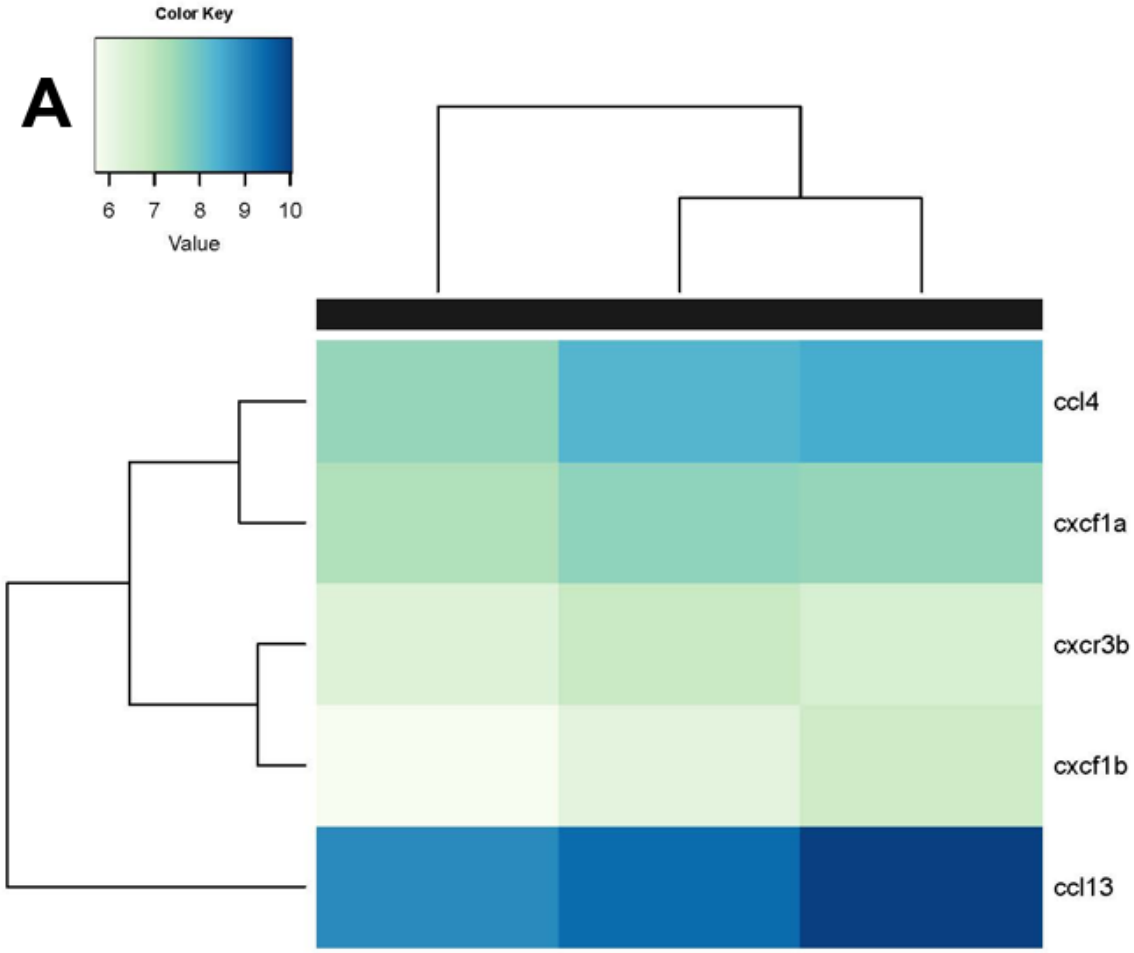


Fig 9. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of all genes identified in rainbow trout posterior kidney of EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.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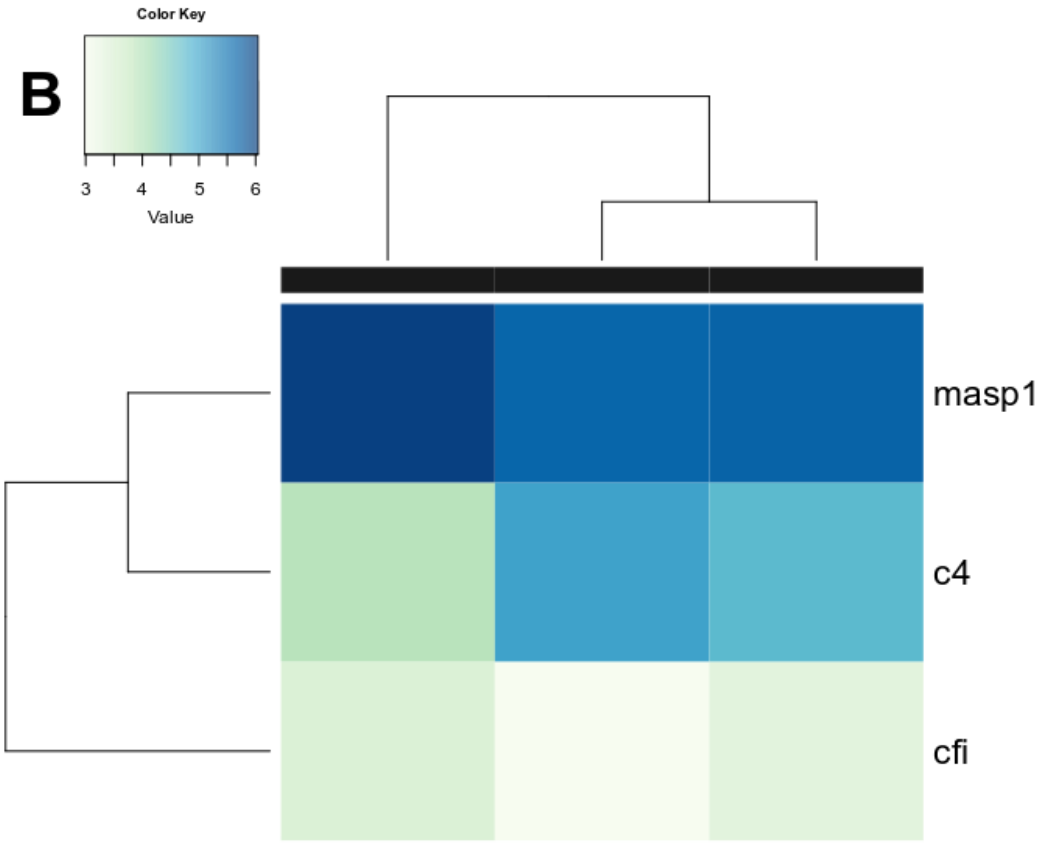
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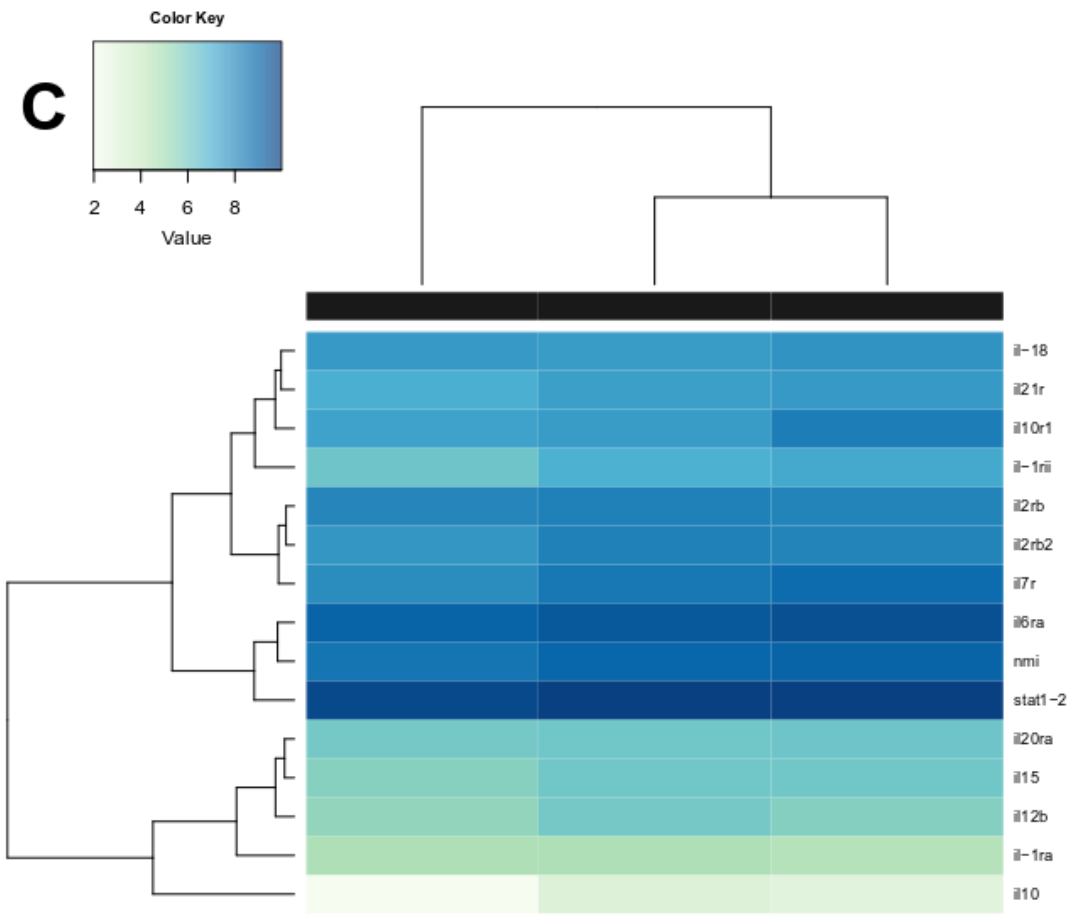
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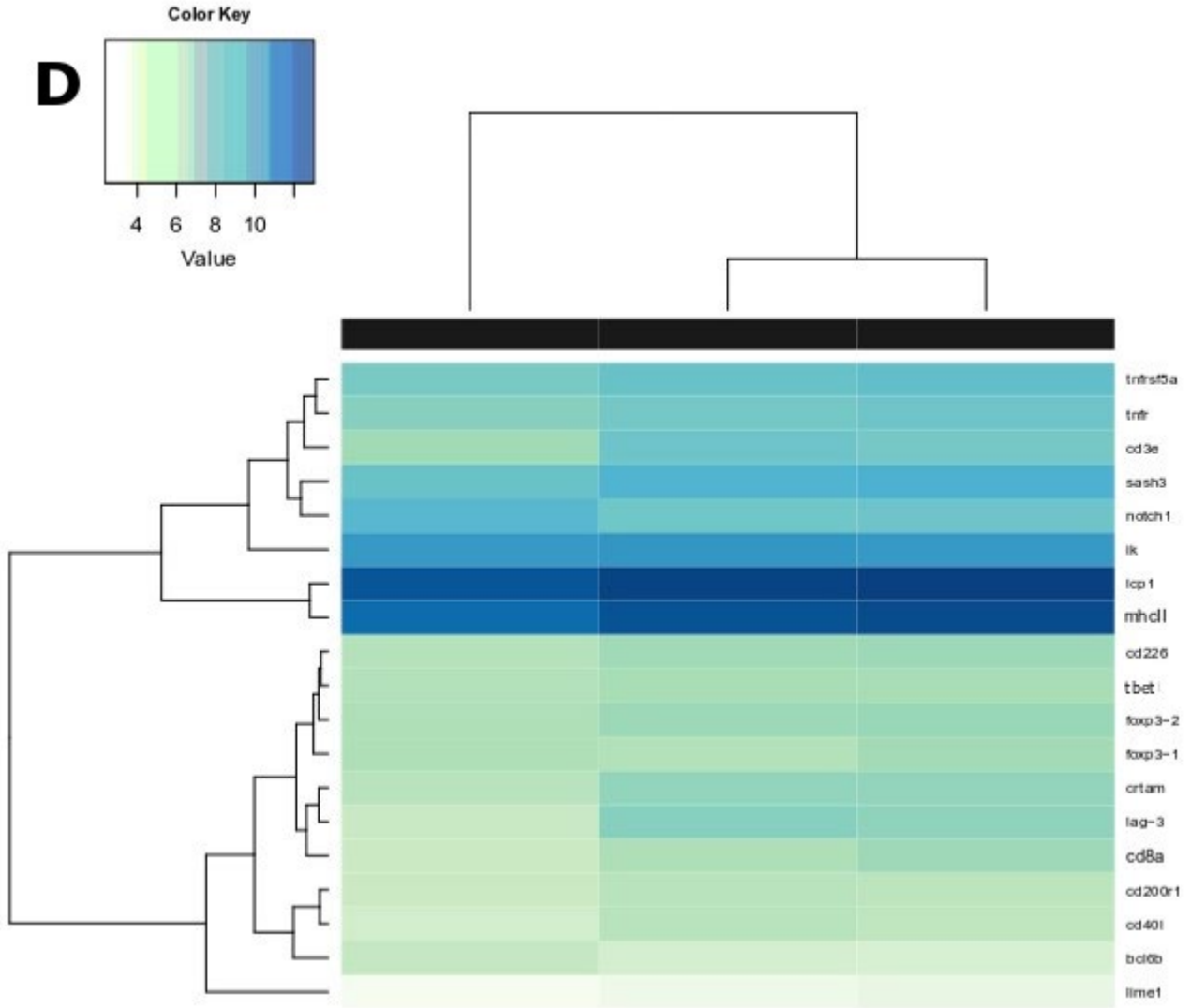

Fig 10. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of A) chemokines; B) complement system; C) cytokines and D) B cell and T cell related genes in the rainbow trout posterior kidney of PKD x EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

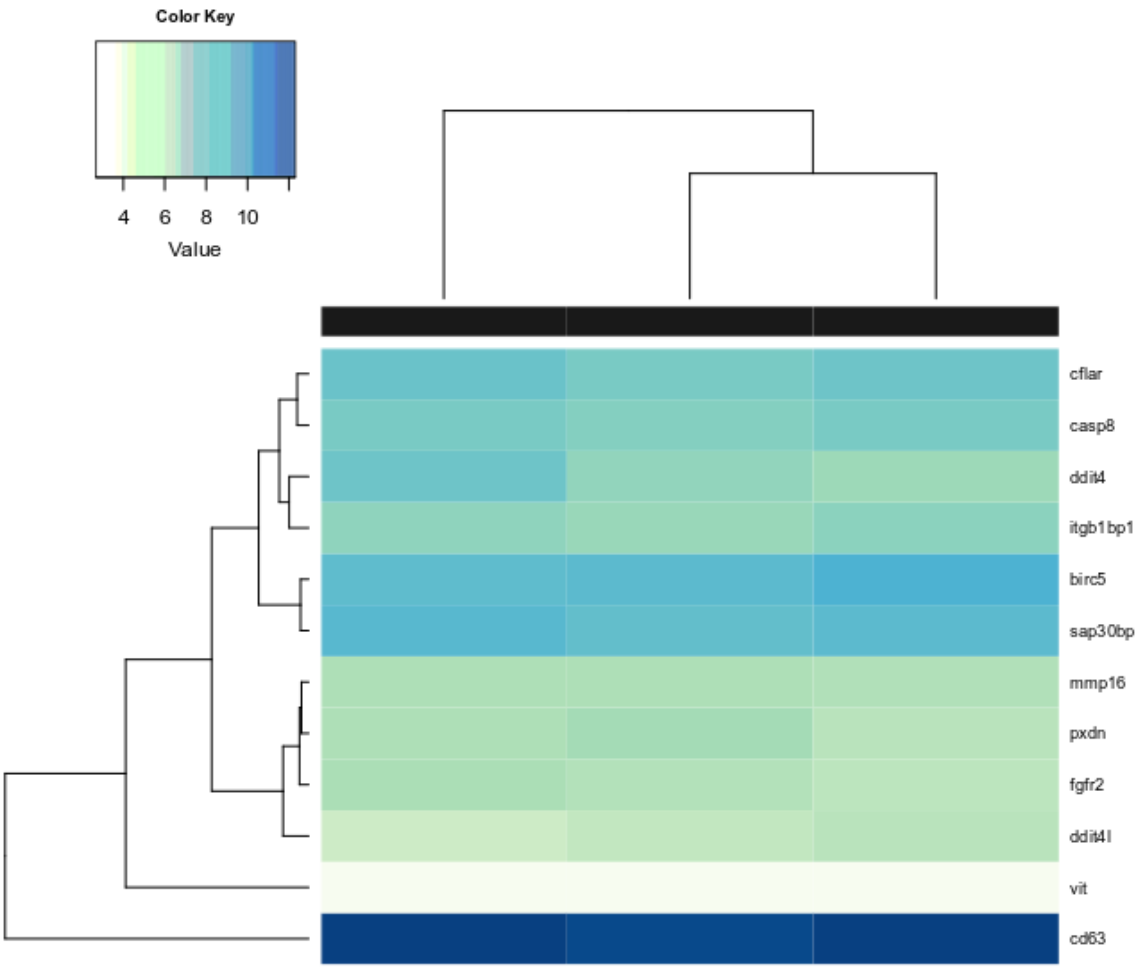

Fig 11. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of inflammation associated genes i.e. genes involved in apoptosis, autophagy and extra cellular matrix (ECM) components in the rainbow trout posterior kidney of PKD x EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

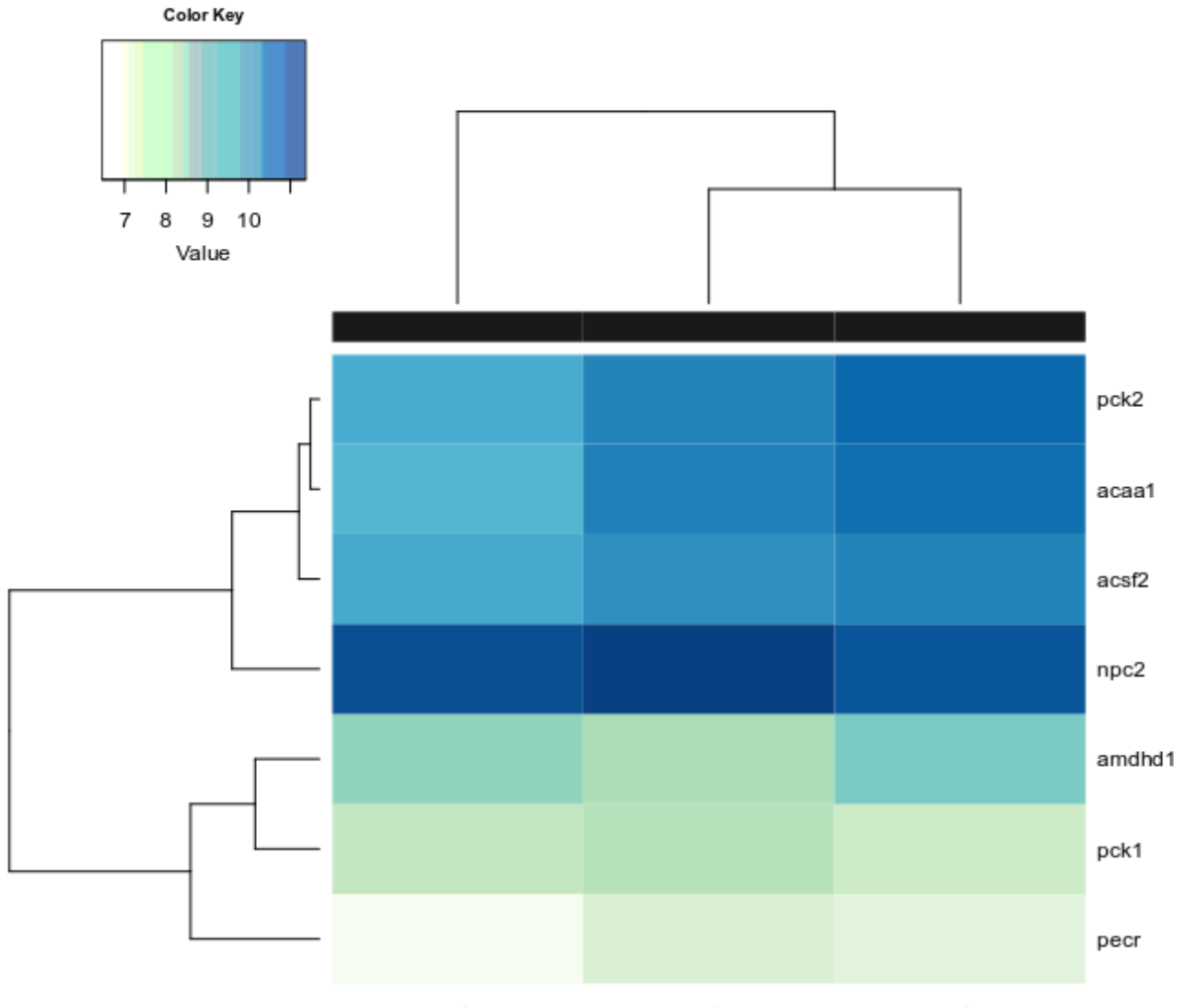

Fig 12. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of genes involved metabolic functions in the rainbow trout posterior kidney of PKD x EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.